Association of Thr715Pro P-Selectin Gene Polymorphism in Diabetic Retinopathy in North Indian Population: A Prospective Clinical Study

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ABSTRACT

Introduction: The aim of this study was to evaluate the role of Thr715Pro P-Selectin gene polymorphism in patients with Diabetic Retinopathy in North Indian population and establish its role in the pathophysiology as an independent factor.

Materials and methods: This is a prospective clinical study conducted on 60 patients at a tertiary care centre in North India over a period of eighteen months. Sixty patients satisfying the inclusion criteria were selected from the Vitreoretina clinic in the department. They were categorised equally in three groups namely Diabetics with diabetic retinopathy (DwDR), Diabetics without diabetic retinopathy (DwoDR), and non diabetics. The non-diabetics group was further divided into healthy controls, Hypertensive Retinopathy (HR) and Non-exudative Age Related Macular Degeneration (NEAMD). All the patients underwent complete ophthalmic evaluation and blood samples were drawn for the genetic study with their informed consent. Data was analysed using SPSS software version 16.

Results: The genotypic analysis between DwDR, DwoDR and the three subgroup of controls comprising of healthy controls, HR and NEAMD showed that Thr715Pro (A/C) polymorphism prevalence was significantly high in DwDR ($p = 0.003$) and DwoDR ($p = 0.003$) compared to healthy controls. No significant difference was noted between DwDR, DwoDR and the HR and NEAMD groups.

Conclusion: Thr715Pro P-Selectin gene Polymorphism could not be established as an independent factor in the pathogenesis of diabetic retinopathy, as its association is found with other systemic diseases which create a prothrombotic state.

Key words: Diabetic retinopathy, Gene polymorphism, Inflammatory markers, P-Selectin gene.

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INTRODUCTION

Diabetes is a lifestyle disorder affecting the whole world, with little difference amongst the developed and the developing nations. The statistical evidence for this metabolic disorder suggests a progressive trend for both the incidence and prevalence of the disease.

Diabetic Retinopathy (DR) arises as a sequel of progressive damage to the retinal microvasculature in response to hyperglycemia. This disturbance of the microcirculation affects retinal nutrition and in turn results in damage to the neural retina. These features have made DR the single most important cause of preventable blindness in adults between 20 to 70 years of age (Cheung et al., 2010).

DR ranks fifth in the list of various studies from 1990-2010, as a cause of moderate to severe visual impairment due to preventable causes (Bourne et al., 2013). Out of the total diabetic population worldwide, a third develop DR, and one third of this population has advanced stages of the disease such as Severe Nonproliferative Diabetic retinopathy (NPDR), Proliferative Diabetic Retinopathy (PDR) or Diabetic Macular Edema (DME) (Yau et al., 2012).

With deeper insights being developed about the disease pathophysiology, inflammation, in the form of elevated cytokine levels in the stored body fat, has been found contributory to the progression of the disease.

DR, which results as a consequence of diabetes, also has an inflammatory component to it. Tumor Necrosis Factor (TNF-α) and Interleukin-1β (IL-1β) have been found to be chronically elevated in these patients. They are identified as inflammatory markers, responsible for the microangiopathic manifestations resulting from endothelial cell and platelet activation.

TNF-α has been found to induce endothelial cell injury and pericyte loss in the early stages of DR, and Deoxyribose Nucleic Acid (DNA) fragmentation on long term follow up , which is quite suggestive of apoptotic cell death and inflammatory injury (Joussen et al., 2009). Interleukin-1β (IL-1β) has been found to induce neuroinflammation in retinal tissues in diabetes. It also causes self stimulation via autocrine and paracrine pathways leading to increased expression of the cytokine levels in retinal tissues. It brings gliotic changes in the long run, thus worsening the pathophysiology (Liu et al., 2012).

Once the inflammatory process sets in, multiple proteins are also released in the circulation, aggravating the disease process. P-Selectin is a protein which is normally released by the platelets and vascular endothelium. But in a state like diabetes, its levels increase mirroring the platelet and endothelial dysfunction. With the evolving advances in genetic research and the emphasis shifting on inflammatory markers and cellular proteins, it is essential to identify

the role played by all the cellular markers and surface proteins in disease progression. Our study aimed at analysing the association of the P-selectin variant with DR, both in quantitative and qualitative manner.

MATERIALS AND METHODS

This is a prospective clinical study done at a tertiary care centre in North India between January 2015 and July 2016. Sixty samples were collected, which included 20 cases each of Diabetics with diabetic retinopathy (DwDR) and Diabetics without diabetic retinopathy (DwoDR) and 20 non diabetic controls, of which 10 were healthy controls and 5 each had Hypertensive Retinopathy (HR) and Non-exudative Age Related Macular Degeneration (NEAMD). All patients were above the age of 40 years. Clearance was obtained from the Committee for Ethical clearance.

The patients having diabetes with and without DR (as defined by the Early Treatment Diabetic Retinopathy Study) were included as cases. Veno-occlusive diseases of the eye, uveitis or glaucoma patients were excluded from being cases in our study. Patients without clinically diagnosed Diabetes or on long term oral steroids for any cause were accepted as the control group.

Basic demographic history was taken, with details pertaining to diabetes duration, medications used and other relevant systemic disorders. Details of fasting and post prandial blood sugar levels, haemoglobin (Hb), glycosylated haemoglobin (HbA1c) were noted.

A complete baseline Ophthalmic evaluation was done consisting of Corrected Distant Visual Acuity (CDVA), 78D slit lamp Biomicroscopy, Indirect Ophthalmoscopy and Optical Coherence Tomography (OCT).

For the detection of gene polymorphism for the Thr715Pro P-Selectin gene, 3 to 5 ml of peripheral blood was collected in Ethylenediamine tetraacetic acid (EDTA) coated vials and sent for Polymerase Chain Reaction (PCR) analysis after due consent from the patients. Primers for PCR amplification were designed utilising the Primer 3 software version 0.4.0 (Rozen S et al., 2000) for Thr715Pro P-Selectin gene polymorphism using sequences from the National Centre for Biotechnology Information (NCBI) Gene database. (Table 1)

Table 1: Primer segments and restriction enzyme used for the immunosorbent assay.

Polymorphism	Reference SNP ID	Reference SNP Alleles	Primer segments	Incision Enzyme
Thr715Pro	rs6136	A/C	FP -5'ATGAACTGCTCCAACCTCTG3'	Hinc II
			RP-5'CCCACATGAAAATTGTACCTT3'.	

Soluble P-Selectin levels were analysed by Enzyme Linked ImmunoSorbent Assay (ELISA) in the pooled plasma samples. ELISA is an immunosorbent assay method utilised for the quantitative measurement of soluble plasma proteins. Here a 96 well coated with P-Selectin antibody was used. Both the standard and sample solutions were added to the well, which were washed off at a later time. Further anti P-Selectin antibody, horseradish peroxidase (HRP) Conjugated Streptavidin and tetramethylbenzidine (TMB) substrate solution were added in consecutive steps. The final colour intensity that developed was quantified at 450nm, which gave a clue to the amount of P-Selectin present in the samples (Figure 1a, b).

The mean value of the data along with standard error of mean as error bars was plotted. Statistical significance ($P < 0.05$) was determined with Student's t test (two-tailed) and nonparametric Analysis of Variance (ANOVA) followed by Bonferroni's multiple comparison test. Results were analysed statistically with the Graph Pad Prism 5.01.

RESULTS

For the assessment of sol P-Selectin levels and the polymorphism of P-Selectin gene, the control group of non diabetics was split in 3 subgroups consisting of 10 Healthy controls and 5 each belonging to HR and NEAMD groups.

Soluble P-Selectin levels (Figure 1) were derived for all the groups, which on ANOVA showed a significant difference of all the four groups (DwDR, DwoDR, HR and NEAMD) with the healthy control (HC) subgroup ($p = 0.005$).

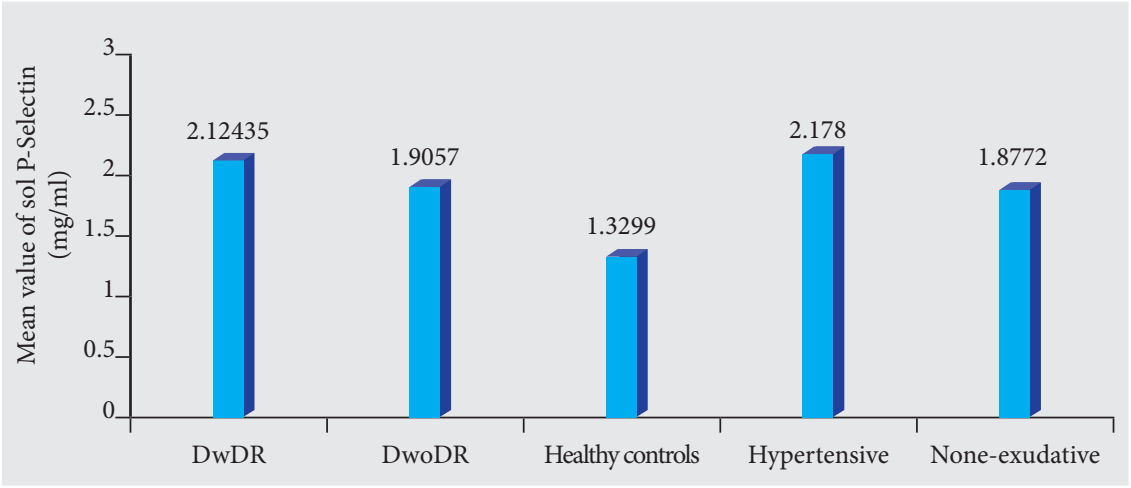

Figure 1: Comparison of mean values of Soluble P-Selectin levels between the groups.

Table 2a: Mean values of Soluble P-selectin levels with standard deviation.

Sol P-selectin	N	Mean	Std. Deviation
Diabetic Retinopathy	20	2.12435	0.677642
Diabetes	20	1.90570	0.537771
Healthy controls	10	1.32990	0.199981
Hypertensive Retinopathy	5	2.17800	0.331910
None-exudative ARMD	5	1.87720	0.193290
Total	60	1.90293	0.579531

Table 2b: ANOVA showed a significant difference of all the groups only with the healthy control subgroup.

Sol P-Sel Levels	Sum of Squares	df	Mean Square	F	P value
Between Groups	4.646	4	1.161	4.211	0.005
Within Groups	15.170	55	.276		
Total	19.816	59			

These four groups had no significant difference among themselves ($p > 0.05$) (Table 2a, b).

On the genotypic analysis between DwDR, DwoDR and the three subgroups of Controls consisting of HC, HR and NEAMD, we found that,

The Thr715Pro (A/C) polymorphism prevalence was significantly higher in DwDR and DwoDR groups than HC with $p = 0.003$ and $p = 0.003$ respectively.

But this prevalence was not significantly different between the DwDR and DwoDR groups ($p = 0.056$).

Also the Control subgroups of HR and NEAMD did not show a statistically significant difference from the DwDR and DwoDR groups for the Thr715Pro (A/C) polymorphism, but there were statistically significant differences amongst the HC and controls with HR and NEAMD at $p = 0.05$ and $p = 0.009$ respectively (Table 3).

The ELISA assay shows the distribution of the allelic frequencies in the collected samples of diabetic population (Figure 2a, b).

Table 3: Genotype characteristics of cases and controls for Thr715 Pro (A/C) Polymorphism.

Genotype	Healthy Controls			Diabetes without Retinopathy			Diabetes with Retinopathy			Non exudative age related macular degeneration			Hypertensive Retinopathy		
	H-W Freq	A.f.	P value	H-W Freq	A.f.	P value	H-W Freq	A.f.	P value	H-W Freq	A.f.	P value	H-W Freq	A.f.	P value
AA	72.25%	A = 85%	0.5678	36%	A = 60%	0.035	33.64%	A = 58%	0.003	30.25%	A = 1155%	0.0097	25%	A = 5 50%	0.0253
AC	25.5%	C = 15%		48%	C = 40%		48.72%	C = 42%		49.5%	C = 9 45%		50%	C = 5 50%	
CC	2.25%			16%			17.64%			20.25%			25%		

H-W Freq: Hardy Weinberg Frequency

A.f: Allelic frequency

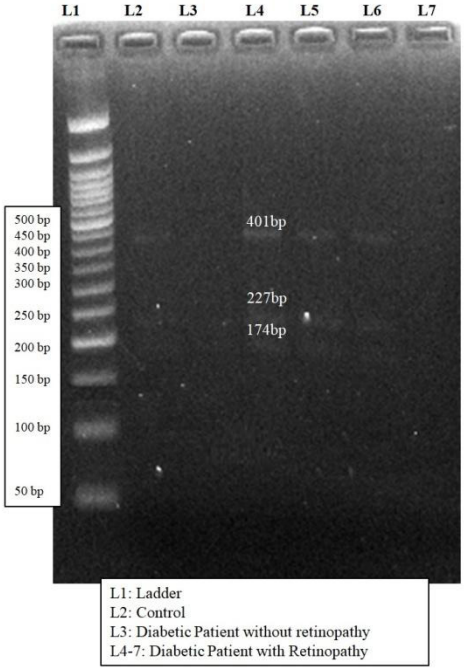

Figure 2a

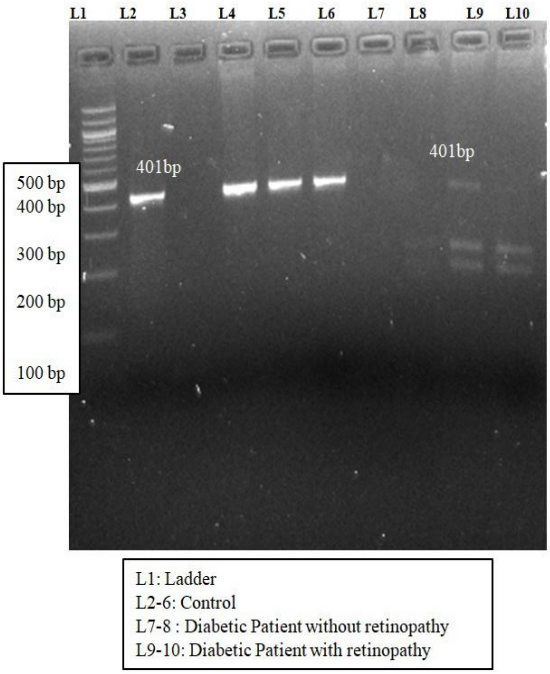

Figure 2b

Figure 2: 3.5% Agarose Gel Electrophoresis for Restriction Digestion of P-Selectin in Control, Diabetic patient without retinopathy and Diabetic patient with Retinopathy. AA homozygous wild type (Single band at 441 bp), GG homozygous mutant (Double bond 227 and 174 bp), AG heterozygous (Triple band: 401 bp, 227 bp and 174 bp).

DISCUSSION

There is a whole body of work analysing genes and its polymorphisms in various systemic and ophthalmic diseases. A battery of specific genes have already been studied in association with DR, namely Aldose Reductase Gene (ALR2) (Abhary et al., 2009), Vascular Endothelial Growth Factor Gene (VEGF) (Sydorova et al., 2005), Receptor for Advanced Glycation End Products Gene (RAGE) (Yang et al., 2013) and Nitric Oxide Synthase Gene (NOS) (Shuzhi et al., 2012).

P-Selectin gene, more commonly known as the SELP gene, is located on chromosome 1. It codes for a 140k Da protein. This protein is stored in the alpha-granules in platelets and Weibel-Palade bodies of the vascular endothelium. It acts as a membrane bound calcium dependent receptor protein, whose main function is to bind to Lewis carbohydrate antigens presented by the cells of the immune system. P-Selectin enables the rolling function of the immune cells on the vascular endothelium, inducing procoagulant microparticle formation (André et al., 2000) and promoting atherosclerosis (Burger et al., 2003). Increased expression of P-Selectin over platelets and soluble levels in plasma has been reported in Diabetes Mellitus and in atherosclerotic diseases.

Numerous studies have dealt with the various polymorphic states of P-Selectin in diseases targeting the macrovasculature, but only a few have addressed the role of Thr715Pro P-Selectin gene polymorphism affecting the microvasculature as in Diabetic Retinopathy.

The Pro715 allele was studied in detail in the Etude Cas-Témoins sur l'Infarctus du Myocarde (ECTIM study) which focussed on its association with myocardial infarction. A total of thirteen polymorphisms were identified, out of which only Thr715Pro polymorphism was related to Myocardial infarction (Kee et al., 2000).

Thr715Pro polymorphism was analysed in association with Venous Thromboembolism (VTE) and was found that it resulted in higher blood levels of soluble P-Selectin augmenting the factors for VTE (Ay et al., 2007). Zalewski et al (2006) studied the role of Thr715Pro polymorphism in the cardiovascular complications of diabetes, but could not reach any positive conclusion. Recently, Kolahdouz et al (2015) in Iran researched the possibility of P-Selectin gene polymorphism with PDR, and found a significant correlation for the rs3917779 allele with PDR (p=0.0001).

Another study of a similar nature was conducted over African Americans having Impaired Glucose Tolerance or Type II Diabetes Mellitus in the Jackson Heart Study, by Penman et al (2015). They measured soluble P-Selectin levels and analysed its role as an independent factor in Diabetic Retinopathy. They found that minor allele homozygotes with rs6128 had significantly lower soluble P-Selectin levels in plasma (p= .046) than the other allelic variants.

No significance was attached to rs6133 and rs3917779.

Our study looked for the association of Thr715Pro polymorphism of the P-Selectin gene in patients having DR, to verify if it could independently be labelled as a factor in the causation of the retinopathy.

We did not find statistical evidence of significance for prevalence of the Thr715Pro variant between the DwDR and DwoDR groups. There was a significant difference among the Healthy controls and both the diabetic groups which justifies the role played by Thr715Pro variant as a factor in the causation of Diabetes. The Allelic frequencies of Thr715Pro variant in the Control subgroups of HR and NEAMD who were free from Diabetes also demonstrated a significant difference from the HC sub group but not with DwDR and DwoDR groups.

The limitation of our study was the small sample size and limited follow up of the patients to see how the variant influences their disease progression. These findings need to be addressed with further prospective studies with a large sample size and a long term follow up.

Long term studies are also needed to identify if it can specifically lead to severe NPDR, PDR or chronic DME, as the burden of visual impairment mostly lies with these subcategories. Thus, we conclude that though Thr715Pro variant was associated with DR, it seems more due to its systemic association with diabetes.

CONCLUSION

Thr715Pro P-selectin variant plays a secondary role in DR, broadly by contributing in platelet aggregation and vascular endothelial damage. This damage is not specifically confined to the retinal vasculature, but also the general vasculature. This effect is not noted in healthy individuals.

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